

Absence of Genetic Differences among G10P[11] Rotaviruses Associated with Asymptomatic and Symptomatic Neonatal Infections in Vellore, India

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ABSTRACT

Rotaviruses (RVs) are leading causes of severe diarrhea and vomiting in infants and young children. RVs with G10P[11] genotype specificity have been associated with symptomatic and asymptomatic neonatal infections in Vellore, India. To identify possible viral genetic determinants responsible for differences in symptomology, the genome sequences of G10P[11] RVs in stool samples of 19 neonates with symptomatic infections and 20 neonates with asymptomatic infections were determined by Sanger and next-generation sequencing. The data showed that all 39 viruses had identical genotype constellations (G10-P[11]-I2-R2-C2-M2-A1-N1-T1-E2-H3), the same as those of the previously characterized symptomatic N155 Vellore isolate. The data also showed that the RNA and deduced protein sequences of all the Vellore G10P[11] viruses were nearly identical; no nucleotide or amino acid differences were found that correlated with symptomatic versus asymptomatic infection. Next-generation sequencing data revealed that some stool samples, both from neonates with symptomatic infections and from neonates with asymptomatic infections, also contained one or more positive-strand RNA viruses (Aichi virus, astrovirus, or salivirus/klassevirus) suspected of being potential causes of pediatric gastroenteritis. However, none of the positive-strand RNA viruses could be causally associated with the development of symptoms. These results indicate that the diversity of clinical symptoms in Vellore neonates does not result from genetic differences among G10P[11] RVs; instead, other undefined factors appear to influence whether neonates develop gastrointestinal disease symptoms.

IMPORTANCE

Rotavirus (RV) strains have been identified that preferentially replicate in neonates, in some cases, without causing gastrointestinal disease. Surveillance studies have established that G10P[11] RVs are a major cause of neonatal infection in Vellore, India, with half of infected neonates exhibiting symptoms. We used Sanger and next-generation sequencing technologies to contrast G10P[11] RVs recovered from symptomatic and asymptomatic neonates. Remarkably, the data showed that the RNA genomes of the viruses were virtually indistinguishable and lacked any differences that could explain the diversity of clinical outcomes among infected Vellore neonates. The sequencing results also indicated that some symptomatic and some asymptomatic Vellore neonates were infected with other enteric viruses (Aichi virus, astrovirus, salivirus/klassevirus); however, none could be correlated with the presence of symptoms in neonates. Together, our findings suggest that other poorly defined factors, not connected to the genetic makeup of the Vellore G10P[11] viruses, influence whether neonates develop gastrointestinal disease symptoms.

Group A rotaviruses (RVs) are a major cause of acute dehydrating diarrhea in infants and children under 5 years of age (1, 2). An assessment of the global impact of RV disease estimated that the virus caused 453,000 deaths in 2008, mostly of children living in sub-Saharan Africa and southeast Asia (3, 4). In India alone, RV infections were estimated to cause 122,000 to 153,000 deaths annually (4). Over the last 40 years, several RV strains have been identified that have a predisposition for replication in neonates, frequently without causing gastrointestinal (GI) disease (5–10). In some cases, the asymptomatic phenotype may reflect the unusual genome constellations that can comprise neonatal virus strains (11–14).

The infectious RV particle is a nonenveloped triple-layered icosahedron that contains 11 segments of double-stranded RNA (dsRNA) (1, 15). The genome directs the expression of 6 structural (VP1 to VP4 and VP6 and VP7) and 6 nonstructural (NSP1 to NSP6) proteins (1). Two of these proteins, the VP7 glycoprotein and the VP4 protease-activated spike protein, form the outer cap-

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sid shell of the RV particle and induce neutralizing antibody responses in the infected host (2). A classification system has been developed that allows assignment of a genotype to each of the 11 RV genome segments (16, 17). The genotype of segments encoding the viral proteins VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/NSP6 is represented by the acronym Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where x is an integer. Whole-genome sequencing has shown that human RVs with the genotype constellation of G1/G3/G4/G9/G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 or G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2 are globally dominant (18–25). Several neonatal RV strains have been identified with overall genotype constellations that are similar to those of the globally dominant strains, with the exception of their P genotypes. For instance, the Australian RV3 neonatal virus has a G3-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1 constellation while the New Delhi neonatal virus 116E has a G9-P[11]-I1-R1-C1-M1-A1-N1-T1-E1-H1 constellation (11, 14, 26). Because such strains are associated with asymptomatic infections and induce immunological protective responses that can ameliorate severe RV diarrhea episodes later in life (8, 11), the RV3 and 116E strains are being pursued as possible vaccine candidates (27–31).

Surveillance studies have determined that G10P[11] RVs are a major cause of neonatal infection in Vellore, India, where the viruses have circulated for more than a decade (32, 33). The persistence of G10P[11] RVs in Vellore may be due, in part, to their widespread contamination of the environment in neonatal nurseries, thus leading to nosocomial infections (33). Unlike the asymptomatic infections caused by some neonatal RVs (e.g., RV3 and 116E), the Vellore viruses have been associated with GI disease in approximately half of the infected neonates (33). The Vellore G10P[11] viruses may not be entirely exclusive to neonates as G10P[11] strains have also been recovered, albeit sporadically, from older children with diarrheal illness (32). In contrast to RV infections in older infants and children, where clinical manifestations typically include watery diarrhea and vomiting, infections by neonatal strains, such as the Vellore G10P[11] virus, can lead to a wide spectrum of other symptoms: abdominal distention, necrotizing enterocolitis, bloody mucoid stools, and intestinal dilation (32–34). It is also noteworthy that while viral load and disease severity can be correlated in older infants and children infected with RVs, viral loads are not significantly different between symptomatic and asymptomatic RV-infected Vellore neonates (35).

Whole-genome sequencing of a G10P[11] RV isolate (N155) recovered in 2003 from a Vellore neonate with GI symptoms revealed that the virus has a genotype constellation of G10-P[11]-I2-R2-C2-M2-A1-N1-T1-E2-H3 (36). Interestingly, G10P[11] RVs are also known to commonly infect calves in India (37, 38). This fact, combined with sequence analysis showing that multiple genome segments of the N155 isolate are highly related to cognate segments of bovine RVs, suggests that the N155 isolate may have originated by reassortment of a bovine G10P[11] RV and a human RV (36, 38).

The discovery that some, but not all, Vellore neonates infected with G10P[11] RVs had GI disease raised the issue of whether multiple, genetically distinct strains of the virus were cocirculating, some which caused illness and some that did not. To address this issue and identify potential virulence determinants (39–42), we used traditional Sanger dideoxynucleotide and next-generation sequencing (NGS) technologies to analyze RNAs contained in stool samples collected from symptomatic and asymptomatic

neonates infected with G10P[11] viruses. The results showed that the G10P[11] viruses were nearly indistinguishable, lacking any differences that could be correlated with symptom status. Although additional enteric positive-strand RNA viruses were detected in some stool samples, none could be causally linked to symptoms. Thus, other poorly defined factors, not connected to the genetic makeup of the G10P[11] viruses or to the presence of other RNA viruses, appear to have a significant role in the development of pathology in infected Vellore neonates.

MATERIALS AND METHODS

Sample collection. Stool samples were collected from neonates admitted in the neonatal nurseries of Christian Medical College (CMC), Vellore, India, during 2003 to 2004 (33). Neonates remaining in the nursery for over 48 h with symptoms of RV-associated GI disease (diarrhea, vomiting, gastroesophageal reflux, GI bleeding, necrotizing enterocolitis, pneumatosis intestinalis, abdominal distension, and/or feed intolerance) were enrolled in the study (33). For each symptomatic neonate enrolled, at least one other neonate was enrolled that had been admitted for 48 h and lacked GI disease symptoms. RV-positive stool samples were identified by enzyme immunoassay (Rota IDEIA; Dako Ltd., United Kingdom). Samples containing G10P[11] RVs were identified by reverse transcription-PCR (RT-PCR) (36). This study was approved by the Institutional Review Board of the CMC, Vellore, India.

Sequencing. Total RNA was prepared from neonatal G10P[11]-positive stool samples with TRIzol (Invitrogen). The RNA was randomly amplified and prepared for NGS using a sequence-independent single-primer amplification (SISPA) method as described elsewhere (43). Briefly, 50 to 200 ng of RNA was combined with dimethyl sulfoxide and a chimeric oligonucleotide containing a 22-nucleotide (nt) barcode sequence with a downstream random hexamer, incubated at 65°C for 5 min, and immediately placed on ice. Reverse transcription was performed by adding the RNA sample to reaction mixtures containing SuperScript III and First Strand buffer (both from Life Technologies), 100 mM dithiothreitol, 10 mM deoxynucleoside triphosphates (dNTPs), and RNase Out (Life Technologies) and incubating at 25°C for 10 min, 50°C for 50 min, and 85°C for 10 min. The cDNA products were denatured at 95°C for 10 min, mixed with (3'→5' exo-) Klenow fragment, and incubated at 37°C for 60 min and then at 75°C for 10 min. Subsequently, the cDNA products were amplified by PCR using *Taq* Gold (Life Technologies) for 35 cycles (denaturation, 30 s at 94°C; annealing, 30 s at 55°C; extension, 48 s at 68°C). PCR mixtures contained primers corresponding to the 22-nucleotide barcode sequence (43). The cDNA products were treated with exonuclease I at 37°C for 60 min, followed by incubation at 72°C for 15 min. Quantities of SISPA products were normalized and pooled into a single reaction mixture that was purified using a PCR purification kit (Qia-gene). The purified cDNA products were then gel purified to select for SISPA products that were 300 to 500 bp in size and submitted for sequencing on Roche 454 GS FLX Titanium and Illumina Genome Analyzer GSIIx platforms. The sequence reads were sorted by barcode, trimmed, and *de novo* assembled into contigs using CLC bio's *de_novo_assembler* program (Qia-gene). To fill in sequence gaps for G10P[11] genome segments, data from NGS were used to design primers that were then used to process viral RNA using Sanger sequencing. The final full-length contigs for G10P[11] viruses were assembled using a combination of data from Sanger, 454, and Illumina sequencing results.

Sequence data analysis. The numbers of individual sequence reads obtained by 454 and Illumina technologies are given in Table 1. The overall project is described elsewhere (<http://www.ncbi.nlm.nih.gov/bioproject/PRJNA220986>), and NGS sequence information is available in the SRA bioinformatics database under GenBank accession numbers SRX504629 (454) and SRX504694 (Illumina). Individual reads were mapped against databases for group A RV ([http://www.ncbi.nlm.nih.gov/nuccore/?term=txid28875\[Organism:exp\]](http://www.ncbi.nlm.nih.gov/nuccore/?term=txid28875[Organism:exp])), Aichi virus and kobuvirus ([http://www.ncbi.nlm.nih.gov/nuccore/?term=txid72149\[Organism:exp\]](http://www.ncbi.nlm.nih.gov/nuccore/?term=txid72149[Organism:exp])), *Astroviridae* ([http://www.ncbi.nlm.nih.gov/nuccore/?term=txid39733\[Organism:exp\]](http://www.ncbi.nlm.nih.gov/nuccore/?term=txid39733[Organism:exp])), salivirus/

TABLE 1 NGS reads for stool specimens recovered from Vellore neonates infected with G10P[11] RVs^a

Neonate	GI symptoms	No. of reads	No. of Illumina reads	Total no. of reads	No. of reads in read class:						
					Group A RV	Aichi virus	Astroviridae	Salivirus/klassevirus	Poivirus	Other virus	Bacteria
N36	No	805	400,528	401,333	384,376	3,339	1,472	0	0	49	9,960
N39	No	620	65,489	66,109	48,334	0	13	0	0	9	1,479
N62	No	1,027	84,470	85,497	82,493	5	0	0	0	16	304
N74	No	9,292	177,240	186,532	64,397	8	0	0	0	14,645	72,108
N121	No	33,411	163,585	196,996	85,509	0	0	0	0	4,043	69,911
N138	No	5,200	221,804	227,004	103,392	0	0	0	0	663	12,175
N190	No	5,517	214,282	219,799	188,717	8	0	0	4	69	22,418
N191	No	33,193	191,125	224,318	36,329	14	0	0	0	268	131,049
N192	No	14,558	223,896	238,454	27,757	9	0	0	0	291	178,840
N203	No	45,486	346,875	392,361	153,093	19	0	0	0	657	135,050
N210	No	7,640	218,292	225,932	38,011	3	0	4	0	706	181,406
N215	No	13,655	139,552	153,207	73,965	0	0	0	0	184	55,635
N223	No	2,488	197,936	200,424	159,418	0	0	0	83	90	35,943
N240	No	223	60,796	61,019	58,563	0	0	0	0	7	1,276
N273	No	4,360	331,278	335,638	178,459	111	0	0	0	1,104	117,351
N284	No	885	175,791	176,676	169,877	0	0	349	0	3	5,689
N291	No	15,219	251,104	266,323	180,311	0	1	2	0	151	74,180
N329	No	9,003	42,066	51,069	8,918	0	0	0	0	18	39,965
N330	No	13,992	83,130	97,122	8,130	6	0	132	0	270	81,420
N375	No	3,354	96,962	100,316	9,950	1	0	0	0	135	87,762
N37	Yes	7,807	257,890	260,697	127,988	4	128,208	0	0	7	644
N83	Yes	2,260	198,636	200,896	185,465	0	0	0	0	337	7,819
N137	Yes	3,205	384,902	388,107	368,168	10,526	0	0	0	117	838
N160	Yes	13,012	185,181	198,193	115,819	1	0	0	0	325	48,237
N184	Yes	1,276	388,344	399,620	390,606	244	0	0	0	20	4,545
N187	Yes	22,058	150,714	172,772	58,433	1	0	0	0	828	65,950
N188	Yes	1,989	119,592	121,581	114,841	0	0	0	0	33	3,845
N196	Yes	20,364	464,194	484,558	1,837	106	0	0	0	778	287,926
N197	Yes	34,114	196,142	230,256	2,281	517	0	0	0	310	217,705
N198	Yes	2,042	500,616	502,658	492,493	17	1	0	0	24	2,062
N212	Yes	11,498	281,050	292,548	184,488	7	0	335	0	291	67,589
N214	Yes	15,461	394,557	410,018	242,927	18	0	0	0	4,443	48,312
N228	Yes	561	437,349	437,910	436,917	73	0	0	0	8	5,014
N232	Yes	229	222,737	272,966	272,803	1	0	0	0	577	4,429
N243	Yes	5,512	679,841	685,353	464,457	3	0	0	0	47	38
N247	Yes	19,053	109,2319	111,1372	259,285	742,512	1	4	0	248	203,517
N259	Yes	8,912	213,353	222,265	14,674	44	0	0	0	369	68,425
N292	Yes	773	346,491	347,264	331,950	0	0	230	0	264	203,244
N313	Yes	1,872	548,839	550,711	528,014	1	0	0	0	3,415	3,871
									6,856	770	148

^a Reads were analyzed by mapping against reference databases, as indicated in Materials and Methods.

TABLE 2 Neonate parameters and other viruses detected in specimens recovered from Vellore neonates infected with G10P[11] RV

RV strain	Neonate characteristic ^a			Other virus in stool specimen ^b				GenBank accession numbers for RV sequences
	Specimen recovery (yr/mo)	Sex	Birthplace	Birth stage	GI symptoms	Aichi virus	Astrovirus	
RV/A/Human-wt/INDDN36/2003/G10P[11]	2003-01	Male	CMC	Term	No	X	X	KC174861-KC174871
RV/A/Human-wt/INDDN39/2003/G10P[11]	2003-01	Male	CMC	Preterm	No	No	No	KC174872-KC174882
RV/A/Human-wt/INDDN62/2003/G10P[11]	2003-02	Male	CMC	Preterm	No	No	No	KC174883-KC174893
RV/A/Human-wt/INDDN74/2003/G10P[11]	2003-03	Female	CMC	Term	No	No	No	KC174894-KC174904
RV/A/Human-wt/INDDN121/2003/G10P[11]	2003-10	Male	CMC	Term	No	No	No	KC174905-KC174915
RV/A/Human-wt/INDDN138/2003/G10P[11]	2003-11	Female	CMC	Term	No	No	No	KC174916-KC174926
RV/A/Human-wt/INDDN190/2004/G10P[11]	2004-01	Male	CMC	Term	No	No	No	KC174927-KC174937
RV/A/Human-wt/INDDN191/2004/G10P[11]	2004-01	Female	CMC	Preterm	No	No	No	KC174938-KC174948
RV/A/Human-wt/INDDN192/2004/G10P[11]	2004-01	Male	CMC	Bordeteline term	No	No	No	KC174949-KC174959
RV/A/Human-wt/INDDN203/2004/G10P[11]	2004-01	Male	CMC	Term	No	No	No	KC174960-KC174969,
RV/A/Human-wt/INDDN210/2004/G10P[11]	2004-02	Male	Outside CMC	Term	No	No	No	KC174970-KC174979,
RV/A/Human-wt/INDDN215/2004/G10P[11]	2004-02	Male	Outside CMC	Term	No	No	No	KC174980-KC174990
RV/A/Human-wt/INDDN223/2004/G10P[11]	2004-03	Female	CMC	Term	No	No	No	KC174991-KC175000,
RV/A/Human-wt/INDDN240/2004/G10P[11]	2004-03	Female	CMC	Preterm	No	No	X	KC1638484
RV/A/Human-wt/INDDN243/2004/G10P[11]	2004-05	Female	CMC	Term	No	X	X	KC175012-KC175022
RV/A/Human-wt/INDDN284/2004/G10P[11]	2004-05	No data	CMC	No data	No	X	X	KC175023-KC175033,
RV/A/Human-wt/INDDN291/2004/G10P[11]	2004-05	Female	CMC	Term	No	No	No	KC1638483
RV/A/Human-wt/INDDN329/2004/G10P[11]	2004-06	Female	CMC	Term	No	No	No	KC175034-KC175044,
RV/A/Human-wt/INDDN330/2004/G10P[11]	2004-06	Male	Outside CMC	Preterm	No	No	No	KC175045-KC175055
RV/A/Human-wt/INDDN357/2004/G10P[11]	2004-07	Female	CMC	Preterm	No	No	No	KC175056-KC175066
RV/A/Human-wt/INDDN37/2003/G10P[11]	2003-01	Female	CMC	Preterm	Yes	X	X	KC175067-KC175077
RV/A/Human-wt/INDDN83/2003/G10P[11]	2003-03	Male	Outside CMC	Term	Yes	X	X	KC175078-KC175088
RV/A/Human-wt/INDDN137/2003/G10P[11]	2003-11	Male	CMC	Term	Yes	X	X	KC175089-KC175099,
RV/A/Human-wt/INDDN160/2003/G10P[11]	2003-12	Female	CMC	Preterm	Yes	X	X	KC1638479
RV/A/Human-wt/INDDN184/2004/G10P[11]	2004-01	Male	CMC	Preterm	Yes	X	X	KC175100-KC175109,
RV/A/Human-wt/INDDN187/2004/G10P[11]	2004-01	Male	CMC	Preterm	Yes	X	X	KC1638478
RV/A/Human-wt/INDDN188/2004/G10P[11]	2004-01	Male	CMC	Term	Yes	X	X	KC175110-KC175120
RV/A/Human-wt/INDDN196/2004/G10P[11]	2004-01	Male	CMC	Term	Yes	X	X	KC175121-KC175131
RV/A/Human-wt/INDDN197/2004/G10P[11]	2004-01	Female	CMC	Preterm	Yes	X	X	KC175132-KC175142
RV/A/Human-wt/INDDN198/2004/G10P[11]	2004-01	Male	CMC	Preterm	Yes	X	X	KC175143-KC175153
RV/A/Human-wt/INDDN212/2004/G10P[11]	2004-02	Female	Outside CMC	Preterm	Yes	X	X	KC175165-KC175175
RV/A/Human-wt/INDDN214/2004/G10P[11]	2004-02	Male	CMC	Preterm	Yes	X	X	KC175176-KC175186
RV/A/Human-wt/INDDN223/2004/G10P[11]	2004-03	Male	CMC	Preterm	Yes	X	X	KC175187-KC175197
RV/A/Human-wt/INDDN228/2004/G10P[11]	2004-03	Male	CMC	Preterm	Yes	X	X	KC175198-KC175208
RV/A/Human-wt/INDDN247/2004/G10P[11]	2004-03	Female	CMC	Preterm	Yes	X	X	KC175209-KC175219
RV/A/Human-wt/INDDN259/2004/G10P[11]	2004-03	Female	CMC	Preterm	Yes	X	X	KC175220-KC175230
RV/A/Human-wt/INDDN259/2004/G10P[11]	2004-03	Male	CMC	Preterm	Yes	X	X	KC175231-KC175241
RV/A/Human-wt/INDDN259/2004/G10P[11]	2004-03	Male	CMC	Term	Yes	X	X	KC175242-KC175252
RV/A/Human-wt/INDDN259/2004/G10P[11]	2004-04	Male	CMC	Preterm	Yes	X	X	KC175253-KC175263
RV/A/Human-wt/INDDN292/2004/G10P[11]	2004-05	Male	CMC	Preterm	Yes	X	X	KC175264-KC175274
RV/A/Human-wt/INDDN313/2004/G10P[11]	2004-05	Female	CMC	Term	Yes	X	X	KC175275-KC175285

^a CMC, Christian Medical College hospital; GI, gastrointestinal.^b Other viruses were detected by BLAST analysis of NGS contigs against the GenBank database. X, stool specimen containing multiple RNA sequences that assembled into a contig matching the genome of the indicated virus.

klassevirus ([http://www.ncbi.nlm.nih.gov/nuccore/?term=txid688449\[Organism:exp\]](http://www.ncbi.nlm.nih.gov/nuccore/?term=txid688449[Organism:exp])), enterovirus ([http://www.ncbi.nlm.nih.gov/nuccore/?term=txid138950\[Organism%3Aexp\]](http://www.ncbi.nlm.nih.gov/nuccore/?term=txid138950[Organism%3Aexp]) with “poliovirus” entered into the definition line), other viruses ([http://www.ncbi.nlm.nih.gov/nuccore/?term=txid10239\[Organism%3Aexp\]](http://www.ncbi.nlm.nih.gov/nuccore/?term=txid10239[Organism%3Aexp]) RefSeq), bacteria (<ftp://ftp.ncbi.nlm.nih.gov/GenBank/genomes/Bacteria/>), and the human genome (<http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/chromFa.tar.gz>). Only reads of greater than 50 nucleotides (after quality and bar-code checks) with identity matches of greater than 80% over at least 90% of the full read were enumerated. Sequence contigs subjected to analysis were, at minimum, 200 nucleotides in length and included two individual reads. Contigs, and unassembled 454 reads, were mapped against the GenBank nonredundant database (<http://www.ncbi.nlm.nih.gov/GenBank>) using the NCBI’s blastn and blastx tools (<http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/blastall>).

The genotypes of RV RNAs were determined using RotaC, version (<http://rotac.regatools.be>) (44).

Sequence alignments. Nucleotide and amino acid sequence alignments and identity values were generated using the ClustalW application of the Geneious Pro (version 6.0) software suite. RV strains with nucleotide and amino acid sequences with close similarity to those of G10P[11] viruses were identified using blastn and blastp (<http://blast.ncbi.nlm.nih.gov>). JProfileGrid (version 2) (<http://www.profilegrid.org>) was used to produce a matrix relating amino acid position, residue identity, and residue frequency (see Dataset S1 in the supplemental material). Identical nucleotide and amino acid sequences were identified using the ElimDuplicates duplicate sequence removal tool (<http://www.hiv.lanl.gov/content/sequence/ELIMDUPES/elimdups/html>).

Nucleotide sequence accession numbers. Accession numbers for the Vellore G10P[11] RVs are given in Table 2.

RESULTS

Sequencing of RNA in stool samples from neonates infected with G10P[11] RVs. Stool samples were collected from 19 symptomatic and 20 asymptomatic neonates infected with G10P[11] RVs. The neonates included both males and females, most were born at CMC hospital, and some were preterm (Table 2). None of these factors (sex, birthplace, and gestational age) is linked to symptom status, confirming results obtained with larger data sets previously (33). RNAs isolated from neonate stool samples were converted to cDNAs and analyzed by both 454 and Illumina NGS technologies, producing more than 12 million reads (Table 1). Due to the large number of reads, it was not feasible to search all reads individually against the complete GenBank database. Instead, reads were mapped against reference databases that included group A rotavirus, Aichi virus, astrovirus, salivirus/klassevirus, other viruses, bacteria, and the human genome (see Materials and Methods). As represented in Fig. 1, 58% of the NGS reads mapped to group A RV, 24% to bacterial, 7% to Aichi virus, and 1% to astrovirus sequences. One percent or less of the sequences reads mapped to salivirus/klassevirus, poliovirus, or other viruses. Of the bacterial reads, approximately 5% mapped to 16S ribosomal sequences (data not shown). Reads mapping to bacteriophage and the *Astroviridae* were common in the “other” virus class; none of the reads in this class correlated with neonate symptom status (data not shown). The reads obtained for each stool sample were assembled *de novo* into sequence contigs; these were searched against the complete GenBank database. Missing sequence information in RV contigs was filled in using Sanger sequencing and primers designed from NGS data. This approach allowed identification of the complete, or nearly complete, open reading frame (ORF) sequences for all 11 genome segments of

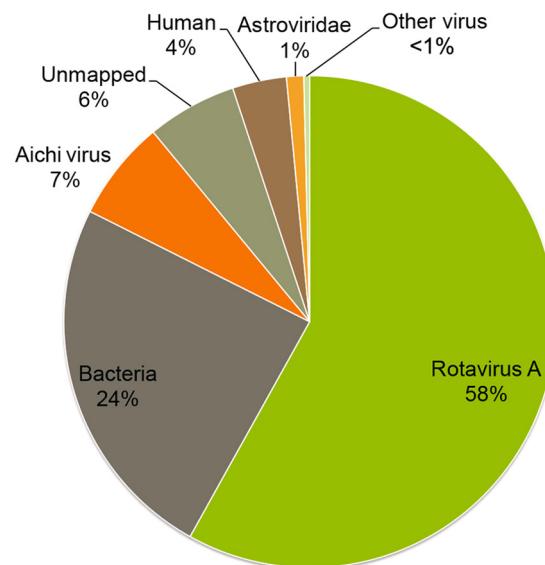


FIG 1 Classes of independent sequence reads generated by NGS analysis of neonatal stool samples. Sequence reads were mapped against the databases indicated in Materials and Methods. The “Other virus” category includes salivirus/klassevirus and poliovirus.

each of the 39 Vellore G10P[11] RVs (accession numbers are given in Table 2).

Vellore G10P[11] RVs share a genotype constellation. Evaluation of genome sequences using the RotaC website (44) indicated that all the Vellore G10P[11] RVs had the same genotype constellation (G10-P[11]-I2-R2-C2-M2-A1-N1-T1-E2-H3), regardless of whether the viruses were isolated from symptomatic or asymptomatic neonates. This genotype constellation was identical to that of the Vellore G10P[11] N155 isolate (36) (Table 3). Another neonatal G10P[11] RV, the I321 strain, was isolated during 1988 to 1991 in Bangalore, India, which is approximately 200 km distant from Vellore (45). Although the genotype constellation of I321 is known only partially, it cannot be same as the Vellore viruses examined in this study, as their NSP2 genotypes differ (N1 and N2, respectively) (Table 3). The complete genomes of other human G10 RVs have been described, but these lack a P[11] VP4 or have a genotype constellation that otherwise differs from that of the Vellore G10P[11] viruses (Table 3): G10P[6]/mani-265/07/IND (46), G10P[8]/163/Vietnam (47), G10P[8]/6717/2002/ARN/NGA (48), G10P[8]/6748/2002/ARN/CIV (48), and G10P[14]/V585/AUS (49). Human RVs have also been described that, although lacking a G10 VP7, have a P[11] VP4 (G6P[11]/SI-B17/SVN) (50) and G9P[11]/116E/IND (14) (Table 3); the genotype constellations of these viruses have additional differences that distinguish them from the Vellore G10P[11] viruses. Despite the numerous reports of human RVs with G10 and/or P[11] genotype specificity, such viruses remain relatively rare compared to viruses with the globally dominant G/P-type combinations: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8] (18, 22).

Vellore G10P[11] RVs likely derived from reassortment of human and bovine RVs. Comparison of the sequences of the Vellore G10P[11] viruses examined in this study with those of the Vellore G10P[11] N155 isolate (36) showed that they were nearly identical, both at the nucleotide and amino acid levels (Table 4). Thus, all these viruses appear to be derived from the same lineage.

TABLE 3 Genotype constellation of Vellore G10P[11] and related rotaviruses^b

Strain/Country	Host	Genotype										
		VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Wa/USA	infant/child	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
DS1/USA	infant/child	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
AU-1/JPN	infant/child	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RV3/AUS	neonate	G3	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
ST3/UK	neonate	G4	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
OH-4/JPN	horse	G6	P[5]	I2	R2	C2	M2	A13	N2	T6	E2	H3
SI-B17/SVN	infant/child	G6	P[11]	I2	R2	C2	M2	A3	N2	T6	E2	H3
116E/IND	neonate	G9	P[11]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Mani-267/07/IND	infant/child	G10	P[6]	I2	R2	C2	M2	A3	N2	T2	E2	H2
6717/2002/ARN/NGA	n.r.	G10	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
6748/2002/ARN/CIV	n.r.	G10	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
163/Vietnam	infant/child	G10	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
N155/IND	neonate	G10	P[11]	I2	R2	C2	M2	A1	N1	T1	E2	H3
Vellore/IND1*	neonate	G10	P[11]	I2	R2	C2	M2	A1	N1	T1	E2	H3
I321/IND	neonate	G10	P[11]	I2	n.r.	n.r.	n.r.	A1	N2	T1	E2	n.r.
V585/AUS	infant/child	G10	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3

^a Genotype for all Vellore neonatal G10P[11] viruses examined in this study.^b n.r., not reported.

Like the results of an earlier study (36), BLAST analysis indicated that several segments (VP7, VP4, VP1, VP2, VP3, VP6, NSP4, and NSP5) of the Vellore strains were closely related to cognate segments of bovine RVs; the sequences of the remaining segments (NSP1, NSP2, and NSP3) were closely related to those of human genotype 1 viruses (Table 4). Consistent with previous proposals (36, 38), these results suggest that the neonatal G10P[11] RVs may have emerged through reassortment of a bovine G10P[11] RV with a human genogroup 1 RV.

G10P[11] RVs associated with asymptomatic and symptomatic infections are genetically indistinguishable. Comparison of the genome segments and encoded proteins of the 39 Vellore G10P[11] viruses showed that they were identical in sequence, or nearly so (96% to 100% identity values). The sequence identity values for viruses that caused asymptomatic or symptomatic infections were not substantially different from the values obtained for all viruses.

Sequence alignments failed to identify any nucleotide in the genome segments of the Vellore G10P[11] viruses that correlated with the presence or absence of disease symptoms (data not shown). Likewise, the proteins of the Vellore viruses lacked any amino acid differences that could be correlated with GI disease symptoms (see Dataset S1 in the supplemental material). Indeed, the protein sequences of G10P[11] viruses recovered from symptomatic neonates were frequently identical to those of viruses recovered from asymptomatic neonates (Table 5). Taken together, these data indicate that the presence or absence of symptoms among the Vellore neonates does not stem from a virulence determinant that differs among the viruses. Thus, differences in the activities of putative RV proteins affecting pathogenesis, including the NSP1 interferon antagonist (51–53), the NSP4 enterotoxin (40, 54, 55), and VP3—an inhibitor of the host oligoadenylate synthetase-RNase L pathway (39)—are not responsible for differ-

TABLE 4 Comparison of the genome segments of Vellore G10P[11] RVs with the cognate segments of N155 and other related strains^a

Genome segment	Genotype	G10P[11] N155 strain			Strain name	Other strain			GenBank accession no.
		Nucleotide identity (%)	Amino acid identity (%)	GenBank no.		Nucleotide identity (%)	Amino acid identity (%)	GenBank accession no.	
VP1	R2	99	99	EU200793	RVA/bovine-tc/USA/NCDV/1971/G6P[1]	94	99	JF693026	
VP2	C2	99	100	EU200794	RVA/bovine-tc/USA/NCDV/1971/G6P[1]	94	99	JF693027	
VP3	M3	98	99	EU200795	RVA/bovine-tc/USA/NCDV/1971/G6P[1]	92	95	JF693028	
VP4	P[11]	99	99	EU200796	RVA/bovine-wt/CHN/DQ-75/2008/G10P[11]	92	93	GU181281	
VP6	I2	99	100	EU200797	RVA/bovine-wt/KOR/KJ9-1/G6P[7]	95	99	HM988974	
VP7	G10	99	100	EU200798	RVA/bovine-wt/ARG/B3326_D_BA/2007/G10P[11]	90	92	KC895795	
NSP1	A1	99	99	EU200799	RVA/human-wt/USA/DC4092/1988/G1P[8]	90	88	KC579470	
NSP2	N1	98	99	EU200800	RVA/human-wt/USA/VU06-07-21/2006/G3P[8]	96	98	JF490909	
NSP3	T1	99	99	EU200801	RVA/human-wt/USA/DC1007/1978/G1P[8]	96	97	KC579594	
NSP4	E2	100	99	EU200802	RVA/bovine-wt/KOR/CBNU-1	95	98	AF166353	
NSP5	H3	98	100	EU200803	RVA/bovine-tc/USA/NCDV/1971/G6P[1]	93	94	JF693036	

^a G10P[11] isolate N184 was used as the reference strain for the Vellore viruses examined in this study.

TABLE 5 Common protein sequences among G10P[11] strains recovered from asymptomatic and symptomatic neonates^a

G10P[11] strain	GI symptoms	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	NSP6
N36	No												
N39	No												
N62	No												
N74	No												
N121	No												
N138	No												
N190	No												
N191	No												
N192	No												
N203	No												
N210	No												
N215	No												
N223	No												
N240	No												
N273	No												
N284	No												
N291	No												
N329	No												
N330	No												
N375	No												
N37	Yes												
N83	Yes												
N137	Yes												
N160	Yes												
N184	Yes												
N187	Yes												
N188	Yes												
N196	Yes												
N197	Yes												
N198	Yes												
N212	Yes												
N214	Yes												
N228	Yes												
N232	Yes												
N243	Yes												
N247	Yes												
N259	Yes												
N292	Yes												
N313	Yes												

^a Common protein sequences were identified using ElimDuplicates (<http://www.hiv.lanl.gov/>). Boxes of the same color represent indistinguishable sequences. A white box represents a unique sequence, with at least one amino acid different from any other.

ences in neonatal symptoms. Rather, our analysis suggests that only a single, genetically nearly identical lineage of the G10P[11] virus circulated in Vellore during 2003 to 2004 and was associated with both symptomatic and asymptomatic infections.

Detection of other enteric viruses in the stool samples of RV-infected neonates. To explore the possibility that disease symptoms among RV-infected Vellore neonates were due to coinfection by other viruses, contigs generated from NGS reads were

mapped by BLAST against all sequences in the GenBank database. The results showed that of the 39 stool samples analyzed, 12 contained RNAs mapping to enteric positive-strand RNA viruses, suggesting that some neonates were coinfected with RV and at least one other virus (Table 2). The mapping results indicated that the coinfecting virus was most commonly Aichi virus, a member of the *Picornaviridae* (56), and, less commonly, salivirus/klassevirus, also a member of the *Picornaviridae* (57), or astrovirus, a member of the *Astroviridae* (58, 59). The location of the contigs relative to the genomes of reference strains is indicated in Fig. 2. The presence of Aichi virus, salivirus/klassevirus, or astrovirus in stool samples may be of importance in defining factors affecting symptoms among the neonates, as surveillance studies have indicated that these viruses can lead to pediatric gastroenteritis (60–64). However, based on evaluation of the data presented in Table 2 using Fisher's exact test (e.g., $P = 0.2351$ for Aichi virus), no statistically significant link existed between any of the coinfecting viruses and symptom status. Thus, other factors are likely responsible for the development of clinical disease among the Vellore neonates. Notably, stool samples that contained RV and Aichi virus RNAs were recoverable over several months in 2003 to 2004 (Fig. 3), suggesting that these viruses cocirculate in Vellore and that coinfections may be relatively frequent.

NGS reads for neonate N223 produced a 236-nt contig that, with the exception of a single residue, perfectly matched that of a

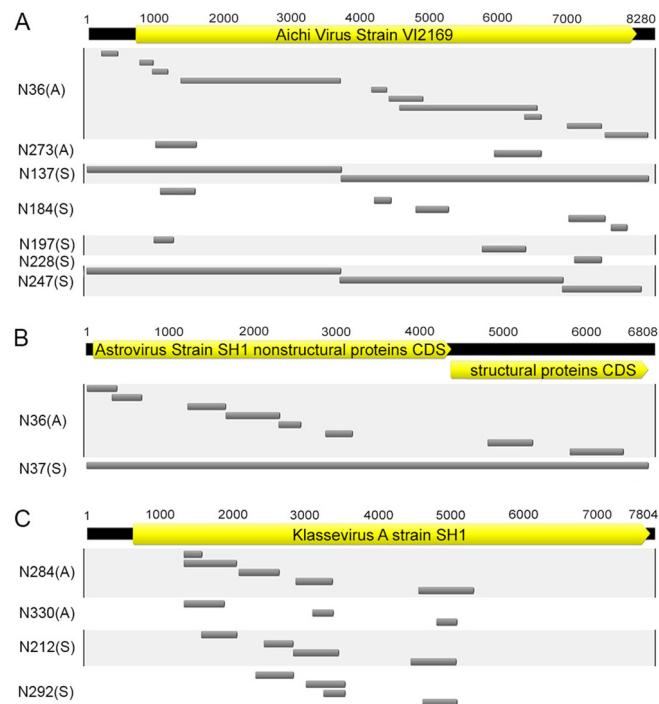


FIG 2 Sequences of other RNA viruses detected in stool samples from Vellore neonates infected with G10P[11] RVs. Sequence contigs generated from NGS reads were used in BLAST searches of the GenBank database. Contigs with matches to Aichi virus (A), astrovirus (B), and salivirus/klassevirus (C) are shown mapped (gray lines) relative to the full-length genome of these viruses. The ORFs of the genome sequences are shown in yellow. The neonate number and category (in parentheses) are indicated to the left as follows: A, asymptomatic; S, symptomatic. The accession numbers of the viruses used to prepare the illustration are GQ927704 (Aichi virus), JQ898343 (salivirus), and FJ375759 (astrovirus). CDS, coding DNA sequence.

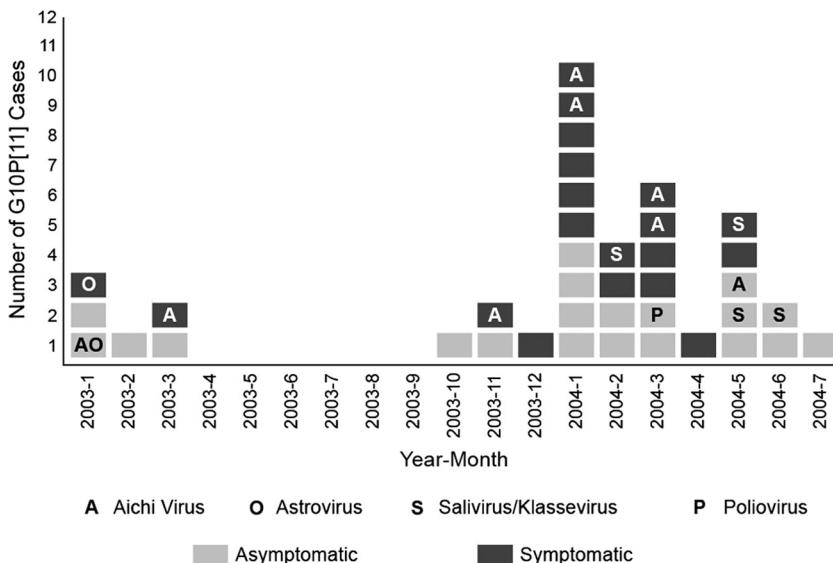


FIG 3 Temporal origin of G10P[11] RVs recovered from Vellore neonates. Blocks represent stool samples containing G10P[11] RV that were sequenced in this study and are colored differently to indicate samples collected from neonates with asymptomatic and symptomatic infections. Samples containing other viral RNAs are coded as indicated.

sequence shared among the Sabin 1, 2, and 3 strains of oral poliovirus vaccine (e.g., residues 6023 to 6258 of Sabin 2; accession number [FJ898290](#)) (Fig. 1). Vellore newborns are given oral poliovirus vaccine at day 0; this may represent the source of poliovirus RNA in this neonate's stool sample.

DISCUSSION

By a combination of next-generation and Sanger sequencing technologies, we have determined that the sequences of G10P[11] RVs infecting symptomatic and asymptomatic Vellore neonates are virtually identical. This result indicates that factors outside the genetic material of the virus determine whether neonates infected with G10P[11] RVs develop symptoms. The nature of these factors is unclear, but their elucidation is important for developing effective antiviral therapies, particularly in neonates which are too young to be afforded protection through vaccination.

Although nearly half of neonatal infections by the Vellore G10P[11] viruses result in GI disease, infections by the Bangalore G10P[11] I321 virus are asymptomatic (45). Because the Vellore and Bangalore viruses have the same G/P-type specificity but differ in their potential to cause GI disease, genome segments of these viruses—other than those encoding VP4 and VP7—may contain differences that affect pathogenesis. Indeed, the I321 genome contains a genotype N1 NSP2 gene while Vellore strains contain a genotype N2 NSP2 gene, hinting at one possible determinant affecting symptom status (Table 3). Previous studies of neonates infected with the Bangalore and Vellore G10P[11] strains produced contrasting results when the subjects were evaluated for protection against subsequent RV infection and disease (65, 66). Specifically, neonates infected with I321-like strains in Bangalore showed evidence of protection, while neonates infected with N155-like strains in Vellore did not (65, 66). Understanding the molecular basis for the differences in the Bangalore and Vellore G10P[11] viruses in inducing neonatal protective responses awaits further sequencing of I321-like strains and should provide

information that is important for engineering asymptomatic vaccine candidates.

NGS analysis indicated that some symptomatic neonates infected with G10P[11] RVs were coinfecting with Aichi virus, astrovirus, and/or salivirus/klassevirus. These viruses commonly infect young children, often without signs of clinical disease (67–71). Coinfections involving RV and other enteric viral or bacterial pathogens have been frequently reported, and there have been a few studies suggesting that coinfections with certain pathogens can alter GI disease severity (63, 72–76). However, little information is available concerning whether coinfection of young children, and particularly neonates, with RV and Aichi virus, astrovirus, or salivirus/klassevirus has an impact on disease symptoms. Nonetheless, our data failed to establish a causal relationship between neonatal symptom status and the presence of a coinfecting positive-strand RNA virus.

Our NGS data did not reveal the presence of norovirus, a leading cause of pediatric gastroenteritis (69, 70, 77, 78), in any of the neonatal stool samples analyzed in this study. This finding was unexpected given an earlier surveillance study indicating that a third of all norovirus-infected Vellore neonates with GI disease were coinfecting with RV (79). Similarly, our NGS data indicated that the Vellore neonates were not infected with orthoreovirus, reovirus, or saffold virus, all RNA viruses that frequently infect children (67, 70, 76, 80–83). At its simplest, the absence of NGS reads for these RNA viruses suggests a possible lack of neonatal exposure or susceptibility to these viruses or a lack of circulation of such viruses in the Vellore area or CMC nurseries. Alternatively, instability or low copy numbers of viral RNAs in stool samples may have also precluded detection by NGS. It is also possible that the cutoff values used in mapping NGS reads against databases were too stringent to identify viruses whose sequences had highly diverged from reference sequences. Because our protocols were directed at detecting RNAs in stool samples, we could have failed

to detect enteric DNA viruses or pathogenic bacteria that affect the symptom status of Vellore neonates.

Whether Vellore neonates develop symptoms is not linked to variations in the genetic material of G10P[11] viruses or, apparently, to how well the virus grows (35). Other factors that may impact disease progression include the level and specificity of maternal anti-RV antibodies acquired by the neonate (84, 85, 86, 87). Alternatively, variability in the physiological maturity and microbiome composition of the neonatal gut may lead to differences in host recognition and response to viral infection (88, 89). It is possible that some aspects of GI disease in RV-infected Vellore neonates may reflect immunopathology triggered by misdirected and/or exaggerated inflammatory responses (90, 91). Such responses have been tied to the development of asthma in respiratory virus infections (92, 93), hemorrhagic fevers in infections with hantavirus (94), yellow fever virus (95), or dengue virus (96), and lung pathology in influenza patients (97, 98). The possibility that some GI disease symptoms in Vellore neonates stem from inappropriate inflammatory responses will require an analysis of the types and levels of inflammatory cytokines and chemokines triggered by RV infections.

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